

## Studies on the Production of Endogenous Pyrogen by Rabbit Monocytes: The Role of Calcium and Cyclic Nucleotides

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Rabbit monocytes stimulated with endotoxin produced endogenous pyrogen, even under conditions of high or low extracellular calcium concentrations. Maximal production occurred when the concentration was in the near-physiological range. Prolonged incubation of cells with a calcium chelator prevented subsequent activation with endotoxin, an effect which was rapidly reversible by re-addition of calcium but not other cations. Addition of small amounts of lanthanum, which acts as a calcium channel blocker, prevented the restoration of pyrogen production, indicating that entry of the added calcium into the monocyte was required. Incorporation of a calcium ionophore into the cell membrane did not stimulate pyrogen production, and no measurable influx or efflux of calcium occurred during stimulation with endotoxin. These observations suggest that a slowly exchangeable calcium pool is necessary for the production of endogenous pyrogen, but that a rise in intracellular calcium is not by itself a necessary or sufficient stimulus. This stands in contrast to other biological systems in which  $Ca^{2+}$  directly couples stimulus and hormone secretion. Incubation of cells with agents shown to increase cyclic 3',5' AMP or cyclic 3',5' GMP levels in monocytes similarly did not stimulate pyrogen production or modulate its production by endotoxin stimulation. Thus, cyclic nucleotides also did not play a detectable role as intracellular messengers in this system. Future work is required to define more clearly the mechanism for the production of endogenous pyrogen, given its marked effects on the immune system through lymphocyte activation and temperature regulation.

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The fever that accompanies infection or inflammation is thought to be mediated by endogenous pyrogen (EP), a soluble product of blood and fixed mononuclear phagocytic cells (MNPC) [1,2]. MNPCs synthesize and release this mediator *in vitro* when stimulated with various agents, including heat-killed bacteria [3,4], products of bacterial cell walls [5-7], and lymphokines released from T cells activated by antigens [8,9] or mitogens [10]. Circulating EP acts upon the thermoregulatory center of the hypothalamus to increase the body's temperature by both reducing heat loss and increasing heat production [11]. There is now evidence that EP is identical to another MNPC product which stimulates T-cell proliferation and activity, lymphocyte-activating factor (LAF) [12-13]. These molecules are collectively referred to as Interleukin 1 (IL-1).

While the production of EP and its subsequent actions have been studied extensively, little is known about intracellular mechanisms of stimulus-secretion coupling in the

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MNPC when EP is produced. Early experiments by Wood and his colleagues showed that rabbit peritoneal exudate cells produced EP "spontaneously" when incubated in saline, but that they became inactive with the addition of calcium ( $\text{Ca}^{2+}$ ) or potassium ( $\text{K}^+$ ) to the incubation medium [14–16]. On the basis of these findings they postulated a role for  $\text{Ca}^{2+}$  in modulating EP release in their system.

In recent years, research in many diverse biological systems has shown an almost universal role for  $\text{Ca}^{2+}$ , alone or in conjunction with cyclic nucleotides, as a second messenger for stimulus-secretion coupling [17]. In the studies reported here we have investigated the role of  $\text{Ca}^{2+}$  and cyclic nucleotides in the production of EP by rabbit blood monocytes stimulated with endotoxin from gram-negative bacteria (lipopolysaccharide, LPS).

## MATERIALS AND METHODS

### *General*

All glassware was sterilized by dry heat at 180°C for two hours to inactivate any pyrogenic contaminants. All reagents used were demonstrated to be pyrogen-free by their inability to stimulate EP production from buffy coat monocytes *in vitro*. Samples from all blood and incubation supernatants were found to be sterile by culture for at least three days at 37°C in thioglycollate broth. Except as noted, all media and reagents were stored at 2°C.

### *Incubation Media*

Most incubations were performed in Hank's balanced salt solution (HBSS) prepared from a 10× concentrate without calcium, magnesium, or bicarbonate (GIBCO). Some experiments were carried out in Eagle's minimum essential medium (MEM) which contained calcium and magnesium (Flow Laboratories). Both media were supplemented with 1.96 g/l sodium bicarbonate, 50,000 u/l penicillin, and 50,000  $\mu\text{g/l}$  streptomycin base. Despite the use of bicarbonate as a buffer, pH often fell as low as 6.9 after incubation.

### *Reagents*

Boivin extracted endotoxin from *E. Coli* 0127.B8 (Difco Laboratories) was prepared as a stock solution of 5 mg/ml in sterile saline (Abbott Laboratories) and diluted to a working solution of 1 ng/ml in saline (for MEM incubations) or in HBSS before each use.

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Certified Grade, Fisher Scientific),  $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$  (Aldrich Chemicals), ethyleneglycol-bis-(B-amino-ethyl ether), and N,N' tetra-acetic acid (EGTA, Sigma Chemical Co.) were sterilized by dry heat at 180°C for two hours before use. Solutions of 100 mM  $\text{CaCl}_2$ , 100 mM  $\text{MgCl}_2$ , and 75 mM  $\text{LaCl}_3$  were prepared in sterile water (Abbott Laboratories). 20 mM EGTA was prepared in HBSS, using 5.6 percent sodium bicarbonate (w/w) to adjust the pH to 7.25. The calcium binding capacity of EGTA, as measured by its competitive ability to bind radioactive  $\text{Ca}^{2+}$ , was not altered by the sterilization process. Ethylenediaminetetra-acetic acid, disodium (EDTA, Disodium Edetate Injection, Riker Laboratories) was diluted to 100 mM in sterile water.

Heparin (Sodium Injection, Eli Lilly and Co.) 1,000 u/ml and Polymyxin B (PMB, Aerosporin, Burroughs Wellcome Co.) 50,000 u/ml were prepared in sterile saline.

Epinephrine (EPI, Adrenalin, Parke-Davis) and propranolol hydrochloride (PROP, Inderal Injectable, Ayerst Labs) were stored as 1 mg/ml solutions in sterile water. N<sup>6</sup>,O<sup>2</sup>-dibutyryl adenosine 3',5'-cyclic monophosphate monosodium salt (db-cAMP, Sigma Chemical Co.), 100 mg/ml, and Staphylococcal enterotoxin B (SE, Sigma Chemical Co.), 1 mg/ml, were prepared in HBSS. Isobutyl-methyl-xanthine (IBMX, Sigma Chemical Co.) was used as a 1 mM solution in sterile saline or culture medium. 8-bromo-guanosine 3',5'-cyclic monophosphate (b-cGMP, Sigma Chemical Co.) was used as a 0.1, 1.0, or 4.0 mM solution. Ionophore A23187 (Calbiochem) 2.0 mM and Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), (prost-13-en-1-oic acid, 11, 15 dihydroxy-9-oxo [11a, 13E, 15S], Sigma Chemical Co.) 1 mg/ml were prepared in 95 percent ethanol and stored at 0°C.

#### *Preparation and Incubation of Cells for EP Production*

Blood was collected into heparinized glass syringes from female five- to six-pound New Zealand white rabbits by cardiac puncture after anesthesia with intravenous sodium pentobarbital (75 mg in 1.5 ml) (Nembutal, Sodium Solution, Abbott Laboratories). In each experiment blood from at least two donors was mixed and centrifuged at 1,000 g for fifteen minutes. The buffy coats were aspirated, resuspended in saline (for experiments in MEM) or HBSS, and washed twice. Total leukocytes were counted twice by hemocytometer, and differential counts were made on 200 consecutive cells from Wright-Giemsa stained smears (mean percentages  $\pm$  SE: lymphocytes 28.8  $\pm$  3.0, granulocytes 61.6  $\pm$  3.2, monocytes 9.6  $\pm$  0.4). Since monocytes are the only important source of EP in blood cells [18], the washed cells were divided into aliquots, each of which contained two to four doses of  $2 \times 10^6$  monocytes mixed with other leukocytes. A standard pyrogenic dose of 100 pg/ml LPS was used in all experiments to stimulate EP production.

In some experiments, to test the effect of extracellular cations upon EP production, the incubation medium was altered by addition of CaCl<sub>2</sub>, MgCl<sub>2</sub>, LaCl<sub>3</sub>, EGTA, or EDTA. In other experiments, cells were preincubated with EGTA for up to three hours before the addition of LPS and/or cations. Some of these EGTA-preincubated cells were incubated further with added cations for up to three hours more before the addition of LPS.

Ionophore A23187, db-cAMP, EPI, PROP, PGE<sub>1</sub>, SE, B-cGMP, IBMX, and PMA were incubated with cells alone, in combination, or with the addition of a standard dose of LPS to test their ability to stimulate EP production or to modify LPS-stimulated EP production. In some experiments PMB (500 u/ml) was added to cell incubations to ascertain whether EP production was due to LPS contamination of potential activators [19].

After all incubations, cells were tested for viability by their ability to exclude eosin dye. Cell viabilities measured by this method were always greater than 90 percent.

In one set of experiments (Ca<sup>2+</sup> pulse), cells suspended in HBSS were incubated with A23187 for five minutes to allow incorporation of the ionophore into the cell membrane. CaCl<sub>2</sub> and EGTA were then added to achieve a buffered free Ca<sup>2+</sup> concentration, and incubation was continued for fifteen minutes. More EGTA was added before beginning the final incubation. This protocol allowed a transient (fifteen-minute) pulse of ionized Ca<sup>2+</sup> into the cells without requiring them to maintain such a high level of internal free Ca<sup>2+</sup> over the extended incubation period.

After addition of all reagents, volumes were standardized so that each dose of  $2 \times$

$10^6$  monocytes was suspended in a total volume of 2.5 ml. The cells were then incubated (5 percent  $\text{CO}_2$ ,  $37^\circ\text{C}$ ) for 20 hours. The suspensions were cleared by centrifugation for ten minutes at 1,000 g, and the supernatants aspirated (average volume 2.3 ml/dose) and assayed for EP by direct injection.

#### *Assay for EP*

The EP content of supernatants was measured by intravenous injection into rabbits. The maximum elevation of temperature above baseline (fever) evoked within one hour, between 0.4 and  $0.8^\circ\text{C}$ , has been shown to be linearly related to the dosage of EP injected [20]. The techniques used to record temperatures were those described by Atkins and Heijn [21], except that rectal temperatures were recorded continuously with a paper recorder (Rustrak TSC, Norwood, MA).

In most experiments, rabbits received a dose of each supernatant in random order. Supernatants were stored at  $2^\circ\text{C}$  but were allowed to warm to room temperature before injection. This technique enabled us to administer injections from a single experiment over a two- to four-day period without appreciable loss of pyrogenicity in the supernatants.

#### *$^{45}\text{Ca}^{2+}$ Tracer Studies*

In order to measure cell-associated  $^{45}\text{Ca}^{2+}$  after addition of ionophore A23187 or of LPS, it was necessary to obtain relatively pure preparations of rabbit monocytes.

*Ionophore Study* A two-step centrifugation procedure using density gradients of Percoll (Pharmacia, Inc.) was used to separate monocytes from heparinized blood (10 u/ml). The method developed by Pertoft et al. [22], for use with human blood cells, was modified to give a better yield with rabbit cells by substituting a 55 percent Percoll solution for the 50 percent solution the authors used in the second centrifugation. After washing the isolated cells twice in MEM, total and differential leukocyte counts were made (differential count mean percentages  $\pm$  SE: lymphocytes  $19.0 \pm 4.4$ , granulocytes  $2.5 \pm 0.5$ , monocytes  $78.5 \pm 4.6$ ).

MEM was added to the cell suspension to bring the concentration of monocytes to  $1.0 \times 10^6/\text{ml}$ . 0.8 ml aliquots of this suspension were incubated in a  $37^\circ\text{C}$  water bath with 0.1 ml of  $^{45}\text{CaCl}_2$  (86 mg Ca/ml, 1–9 mCi/ml, Radioactive Center, Amersham, England) that had been diluted 1:100 with MEM, and 0.2 ml of a 2 mM solution of ionophore A23187 or of MEM (control). At timed intervals, 50  $\mu\text{l}$  aliquots were removed and passed through a 0.45  $\mu\text{m}$  pore size filter (Type HA, Millipore Corp.). Each filter was immediately washed twice with 1 ml aliquots of ice-cold MEM and then placed in a scintillation vial with 4 ml of Hydrofluor (National Diagnostics). Total counts were measured by resuspending 50  $\mu\text{l}$  of the unfiltered incubation medium in 4 ml of Hydrofluor. Each sample was counted for one minute on a scintillation counter (Model LS 3133P, Beckman Instruments).

Cells isolated by Percoll produce EP spontaneously. That activity, however, does not prevent the rise in intracellular calcium which correlates with incorporation of ionophore into the membrane.

*LPS Study* As monocytes isolated with Percoll produced EP without further stimulation, it was necessary to adapt the method described above still further. This modification involved substituting a mixture of Ficoll and Hypaque for Percoll in creating gradients of the same densities as used previously. Cells obtained from the second centrifugation were again washed twice in MEM before making total and

TABLE 1  
Mean Fevers Produced by Supernatants from Monocytes Incubated With or Without LPS in HBSS with Various Concentrations of CaCl<sub>2</sub>

LPS (pg/ml)	CaCl <sub>2</sub> (mM)	ΔT (±SE) (°C)	Number of Recipients
0	2.0	0.0	2
100	0	0.675 ± 0.063 <sup>a</sup>	4
100	0.5	0.900 ± 0.087 <sup>ab</sup>	3
100	1.0	0.788 ± 0.097 <sup>b</sup>	4
100	2.0	0.363 ± 0.080 <sup>ab</sup>	4

<sup>a</sup>*p* < 0.025 by paired *t*-test

<sup>b</sup>*P* < 0.05 by paired *t*-test

<sup>ab</sup>Difference not statistically different from incubations with 0 or 1.0 mM CaCl<sub>2</sub>

differential leukocyte counts (differential count mean percentage ± SE: lymphocytes 12 ± 1, granulocytes 3 ± 1, monocytes 85 ± 2).

MEM was added to the cell suspension to bring the concentration of monocytes to 2.3 × 10<sup>6</sup>/ml. 0.9 ml aliquots were incubated in a 37°C water bath with 0.05 ml of the <sup>45</sup>CaCl<sub>2</sub> solution described above. After two hours, 100 pg LPS and/or 500 u PMB were added in 0.1 ml MEM. 50 μl aliquots were removed at timed intervals and filtered, washed, and assayed, as above.

#### *Calculation of Free Calcium Concentration [Ca<sup>2+</sup>]*

When Ca<sup>2+</sup> and chelators were mixed in solution, the free calcium concentration [Ca<sup>2+</sup>] was calculated, using the method of O'Sullivan, to determine the "apparent" stability constants of chelate complexes at pH 7.30, the initial incubation pH, from the published "absolute" values of the constants [22]. The calculated values for the stability constants were 3.98 × 10<sup>7</sup> M<sup>-1</sup> for Ca-EDTA complexes and 1.896 × 10<sup>7</sup> M<sup>-1</sup> for Ca-EGTA complexes.

## RESULTS

### *Effects of Free Calcium on EP Production*

Buffy coat cells were incubated with LPS in HBSS with or without added CaCl<sub>2</sub>. LPS stimulated EP production in all incubations, but the greatest response occurred with the addition of 0.5 mM CaCl<sub>2</sub> to the incubation medium (Table 1).

When other cells were incubated with LPS in an incubation medium prepared by diluting MEM 1:2 with sterile saline, EP was likewise produced despite the addition of CaCl<sub>2</sub> or EDTA to alter the Ca<sup>2+</sup> (Table 2). The maximum EP production occurred with a calculated [Ca<sup>2+</sup>] of 0.4 mM.

### *Effects of EGTA Preincubation on Subsequent EP Production*

Cells were preincubated with 5 mM EGTA for zero to three hours before the addition of LPS. Twenty-hour supernatants were assayed for EP, and the mean fevers of EGTA-preincubated cells are expressed as percentages of the responses from cells activated with LPS without preincubation after control responses (cells incubated alone) were subtracted from both (Fig. 1). The mean fever for cells not preincubated with EGTA was 0.80 ± 0.05°C (SE). Preincubation with EGTA resulted in substantial

TABLE 2  
Mean Fevers Produced by Supernatants from Monocytes Incubated with 100 pg/ml LPS in MEM-Saline Solution with Various Concentrations of EDTA and Ca<sup>2+</sup>

EDTA (mM)	EDTA <sup>a</sup> (Calcium)	Calculated Free Calcium Concentration (mM)	ΔT (±SE) <sup>e</sup> (°C)
0	0	1.9 <sup>b</sup>	0.767 ± 0.081 <sup>d</sup>
0.50	0.56	0.4	1.25 ± 0.04 <sup>d,e,f</sup>
1.00	1.11	2.3 × 10 <sup>-4</sup>	0.650 ± 0.065 <sup>e</sup>
2.00	2.22	2.1 × 10 <sup>-5</sup>	0.738 ± 0.115 <sup>f</sup>

<sup>a</sup>Based on a total calcium concentration of 1.8 mM in MEM, and a resulting total calcium concentration of 0.9 mM in our incubation medium which was MEM diluted 1:2 with saline

<sup>b</sup>1.0 mM CaCl<sub>2</sub> was added to this solution

<sup>c</sup>There were four recipients for each supernatant.

<sup>d</sup> $p < 0.025$  by paired  $t$ -test

<sup>e</sup> $p < 0.005$  by paired  $t$ -test

<sup>f</sup> $p < 0.005$  by paired  $t$ -test

inhibition of LPS-stimulated EP production, and the degree of inhibition increased with prolongation of the preincubation period.

#### Reversibility of EGTA Effects

Inhibition of LPS-induced EP production by cells preincubated for three hours with 5 mM EGTA was partially reversed when 5 mM CaCl<sub>2</sub> was added to the incubation medium simultaneously with LPS (Fig. 2). Lesser amounts of CaCl<sub>2</sub> were not sufficient to restore EP production. A second preincubation of cells with CaCl<sub>2</sub> for up to three hours after the initial treatment with EGTA did not affect the ultimate release of EP compared to that seen when CaCl<sub>2</sub> and LPS were added together (data not shown).

Substitution of 5 mM MgCl<sub>2</sub> or LaCl<sub>3</sub> for CaCl<sub>2</sub> did not restore EP production following EGTA preincubation (Fig. 3). Furthermore, incubation of cells with 0.1 mM MgCl<sub>2</sub> or LaCl<sub>3</sub> for three hours after the initial treatment with EGTA prevented EP production when LPS and 5 mM CaCl<sub>2</sub> were subsequently added.

#### Effects of Ionophore A23187 on EP Production

Cells were incubated for 20 hours with 0.20 μM A23187 in HBSS to which 25 μM CaCl<sub>2</sub> or MgCl<sub>2</sub> or both had been added. Under these conditions the ionophore did not stimulate EP production (Table 3). Cells incubated with both 0.20 μM A23187 and 100 pg/ml LPS released EP, though in amounts that were significantly lower than those produced in the absence of A23187 ( $p < 0.05$  by paired  $t$ -test).

In another set of experiments, we attempted to produce a pulse of intracellular calcium influx by incubating cells for five minutes in HBSS with 0.20 or 2.00 μM A23187, followed by the addition of Ca<sup>2+</sup> and EGTA to produce buffered solutions with free calcium concentrations of 0.37 or 2.30 μM. Incubations were continued for fifteen minutes, followed by the addition of either more EGTA to reduce the [Ca<sup>2+</sup>] to 0.02 μM or more HBSS to keep the [Ca<sup>2+</sup>] stable. Twenty-hour supernatants did not show any EP activity (data not shown), although an aliquot of the same cells incubated with LPS produced EP (mean fever 1.08°C;  $n = 2$ ).

To confirm that added A23187 did interact with cells during incubation, rabbit

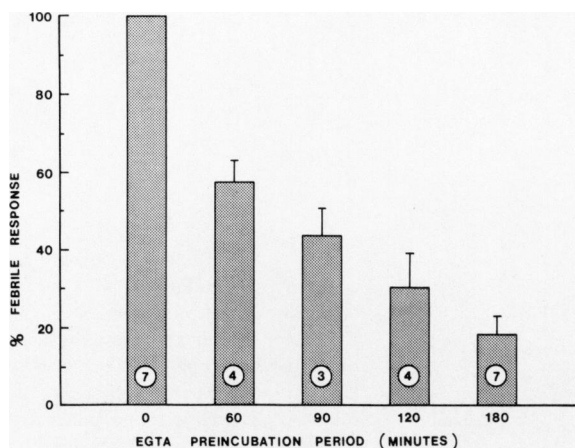


FIG. 1. Mean fevers ( $\pm$ SEM) produced by supernatants from  $2 \times 10^6$  monocytes incubated for 20 hours with 100 pg/ml LPS after preincubation of cells with 5 mM EGTA for 0–180 minutes, expressed as a percentage of the response seen when cells were not preincubated with EGTA. Numbers of recipients for each supernatant are given in parentheses.

mononuclear cells isolated on Percoll gradients were incubated with  $^{45}\text{Ca}^{2+}$  in the presence and absence of  $0.36 \mu\text{M}$  A23187. Cells treated with ionophore showed an increase in cell-associated counts during the first 30 minutes of incubation, while the counts in untreated cells did not increase during a two-hour incubation period (Fig. 4).

#### $^{45}\text{Ca}^{2+}$ Tracer Studies of LPS Stimulation

Rabbit mononuclear cells isolated on Ficoll-Hypaque gradients were incubated with  $^{45}\text{CaCl}_2$  for two hours to allow equilibration before the addition of 100 pg/ml LPS and/or 500 u/ml PMB. No flux of  $\text{Ca}^{2+}$  could be demonstrated by changes in cell-associated  $^{45}\text{Ca}^{2+}$  upon the addition of LPS (data not shown). Separate aliquots of cells were incubated with or without LPS, and the 20-hour supernatants were assayed for EP. The aliquots incubated with LPS produced EP (mean fever  $0.5^\circ\text{C}$ ;  $n = 2$ ), while the aliquots without LPS were non-pyrogenic.

#### Effects of cAMP on EP Production

Buffy coat cells incubated 20 hours with 0.01 mg/ml EPI and/or 0.01 mg/ml PROP produced no detectable EP (data not shown). Direct intravenous injection of these drugs at this dose produced no changes in body temperature of test rabbits. To investigate the effects of EPI and PROP as modulators of an independently stimulated response, cells were incubated with 100 pg/ml LPS with or without the addition of EPI or PROP (0.01 mg/ml). These drugs did not affect the response of monocytes to LPS (Table 4). Similarly, we found that  $14 \mu\text{M}$  PGE<sub>1</sub> did not stimulate EP production when incubated with cells, nor did it affect LPS stimulation of EP production.

$10 \mu\text{g}/\text{ml}$  SE stimulated release of EP sufficient to cause a mean fever of  $0.80 \pm 0.05^\circ\text{C}$ , but addition of 500 u/ml PMB before incubation abolished the effect. Similarly, supernatants from cells incubated with 3 mM db-cAMP produced fevers of  $0.60 \pm 0.19^\circ\text{C}$ , but this was prevented by including 200 u/ml PMB in the incubation medium.

#### Effects of cGMP on EP Production

Incubation of cells with PMA (an agent which stimulates endogenous cGMP production) in a concentration of 100 ng/ml for 24 or 48 hours did not cause EP

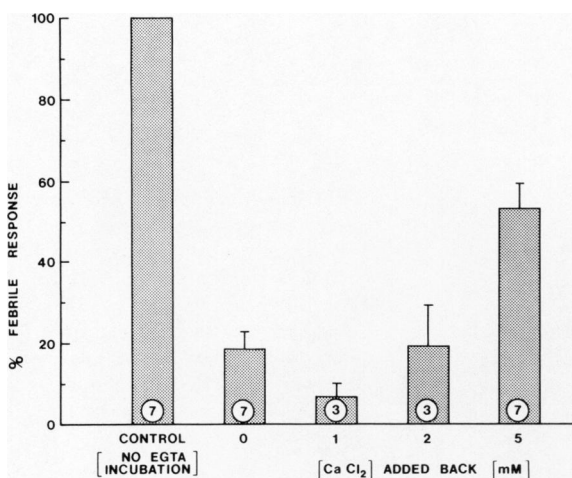


FIG. 2. Mean fevers ( $\pm$ SEM) produced by supernatants from  $2 \times 10^6$  monocytes preincubated with 5 mM EGTA for three hours before the addition of LPS (100  $\mu$ g/ml) alone or with CaCl<sub>2</sub>, expressed as a percentage of the response when cells were not pretreated with EGTA. Numbers of recipients are given in parentheses.

production (Table 5). This concentration of the same preparation of PMA was active, however, in stimulating the metabolic oxygen burst in neutrophils and monocytes [Nauseef W, Metcalf J: Personal communication].

1 mM B-cGMP stimulated production of EP by cells, but this was suppressed by addition of 500 u/ml PMB during incubation. Similar results were obtained with 1 mM IBMX, a phosphodiesterase inhibitor, with and without B-cGMP (Table 5).

## DISCUSSION

Using a standard dose of LPS as a pyrogenic stimulus, we found that rabbit blood monocytes can produce EP in media with free calcium concentrations in the  $10^{-8}$  to  $10^{-3}$  M range. As in Wood et al.'s studies [14–16] we found that EP production was inhibited at greater than physiological concentrations of free calcium. That we saw more inhibition in HBSS than in MEM can be attributed to calcium-binding components in the latter medium, notably succinic acid and the amino acids present, which reduce the concentration of free calcium. MEM also contains more phosphate and sulfate anions, which complex with calcium as well. In both media, however, we

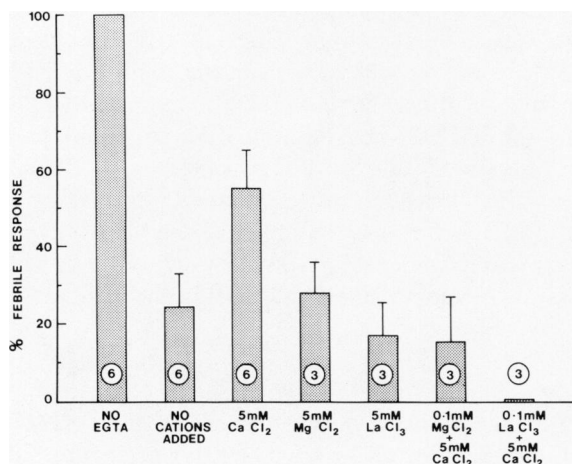


FIG. 3. Mean fevers ( $\pm$ SEM) produced by supernatants from  $2 \times 10^6$  monocytes preincubated with 5 mM EGTA for three hours before addition of 100  $\mu$ g/ml LPS with or without the addition of CaCl<sub>2</sub>, MgCl<sub>2</sub>, or LaCl<sub>3</sub>. To two aliquots of cells, 0.1 mM MgCl<sub>2</sub> or LaCl<sub>3</sub> was added after the EGTA preincubation, and cells were incubated for an additional three hours before the addition of LPS. Numbers of recipients are given in parentheses.



TABLE 3  
 Mean Fevers Produced by Supernatants from Monocytes Incubated with 0.20  $\mu\text{M}$  A23187 and/or 0.25 ng LPS in the Presence of 25  $\mu\text{M}$   $\text{CaCl}_2$  and/or  $\text{MgCl}_2$  in HBSS

Potential Activator	Cations Present	$\Delta T (\pm \text{SE})^a$ ( $^\circ\text{C}$ )
None	$\text{Ca}^{2+}, \text{Mg}^{2+}$	$0.16 \pm 0.01$
A23187	$\text{Ca}^{2+}$	$0.15 \pm 0.02$
A23187	$\text{Mg}^{2+}$	$0.08 \pm 0.04$
A23187	$\text{Ca}^{2+}, \text{Mg}^{2+}$	$0.14 \pm 0.06$
A23187 + LPS	$\text{Ca}^{2+}$	$0.31 \pm 0.05^b$
LPS	—	$0.68 \pm 0.13^b$

<sup>a</sup>There were four recipients for each supernatant.

<sup>b</sup> $p < 0.05$  by paired  $t$ -test

found maximum EP production with a calculated free  $[\text{Ca}^{2+}]$  of 0.4–0.5 mM. This value is lower than the average value of 1.15 mM free  $\text{Ca}^{2+}$  Moore measured in the serum of normal volunteers [24]. Although all buffy coat preparations were washed twice in  $\text{Ca}^{2+}$ -free solutions (probably containing  $\mu\text{M}$  levels of  $\text{Ca}^{2+}$  as a trace contaminant) significant amounts of  $\text{Ca}^{2+}$  may have remained bound to cell membranes. This would increase the actual amount of  $\text{Ca}^{2+}$  present and, perhaps, raise the local concentration of free  $\text{Ca}^{2+}$ , in addition. The fall in pH seen during the course of incubation, to as low as 6.9, would increase free  $\text{Ca}^{2+}$  further by decreasing the stability of calcium complexes. In our HBSS incubations, the difference in fever stimulated by EP generated with 0.5 and 1.0 mM free calcium was not statistically significant ( $p > 0.05$  by paired  $t$ -test), and no comparable incubation with 1 mM free  $\text{Ca}^{2+}$  was performed in MEM. Therefore, we cannot exclude the possibility that EP production is maximal at approximately physiological concentrations of free  $\text{Ca}^{2+}$ .

While monocytes could be stimulated to produce submaximal amounts of EP with extremely low levels of free extracellular  $\text{Ca}^{2+}$ , preincubation of cells with a  $\text{Ca}^{2+}$  chelator inhibited subsequent stimulation of EP production. The degree of inhibition was related to the time of preincubation. It was partially reversed by later addition of  $\text{Ca}^{2+}$ , but not other cations, to the system. In fact, incubation with small amounts of

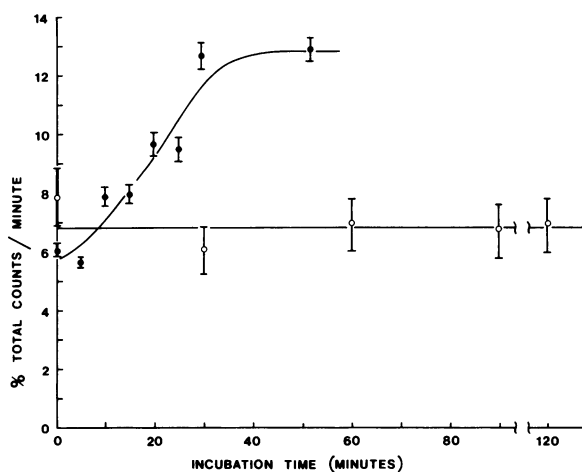


FIG. 4. Counts per minute of filtrates of 50  $\mu\text{l}$  aliquots of cells incubated with (●) or without (○) 0.36  $\mu\text{M}$  A23187, expressed as a percentage of the total counts in an unfiltered 50  $\mu\text{l}$  aliquot. Total counts were  $46,043 \pm 1,284$  for the cells incubated with ionophore and  $57,516 \pm 7,137$  for the cells incubated alone. Standard errors are shown.

TABLE 4  
 Mean Fevers Produced by Supernatants from  $2 \times 10^6$  Monocytes  
 with 0.01 mg/ml EPI and/or 0.01 mg/ml PROP in the Presence of  
 100 pg/ml LPS

Activator(s)	$\Delta T (\pm SE)^a$ (°C)
LPS	$0.86 \pm 0.10$
LPS + EPI	$0.84 \pm 0.09$
LPS + PROP	$0.73 \pm 0.15$
LPS + EPI + PROP	$0.76 \pm 0.10$
PGE <sub>1</sub>	$0.10 \pm 0.06$
LPS + PGE <sub>1</sub>	$0.78 \pm 0.07$

<sup>a</sup>There were four recipients for each supernatant.

Mg<sup>2+</sup> or La<sup>3+</sup> prevented the reversal of inhibition by later addition of Ca<sup>2+</sup>. However, the prolonged incubation of cells depleted of Ca<sup>2+</sup> during these experiments may have had other effects which prevented subsequent stimulation. These observations suggest that there is a pool of Ca<sup>2+</sup> important to the process of pyrogenic stimulation and/or EP production which is somehow protected from rapid equilibration with extracellular Ca<sup>2+</sup>. This pool may be bound to the plasma membrane or may be sequestered in an intracellular site, such as the mitochondria or endoplasmic reticulum. Macrophages have a membrane-bound Mg<sup>2+</sup> and ATP-dependent Ca<sup>2+</sup> pump which transports free Ca<sup>2+</sup> from the cytoplasm to the extracellular surface [25], and over time the action of this pump could deplete the intracellular stores of Ca<sup>2+</sup> if reentry is prevented by formation of EGTA-Ca<sup>2+</sup> complexes extracellularly. Borle has shown that mitochondria lose Ca<sup>2+</sup> slowly with a half-time of 152 minutes [26], while Mela and Chance have shown that mitochondrial Ca<sup>2+</sup> uptake has a half-time of eight seconds [27]. These observations are consistent with our results showing that a lengthy preincubation with EGTA is required to deplete the necessary Ca<sup>2+</sup> pool, but that the pool is replenished rapidly as evident by restoration of the response when Ca<sup>2+</sup> is added together with the pyrogenic stimulus. Since nearly complete depletion of the intracellular pool is required to inhibit EP release, Ca<sup>2+</sup> does not appear to be the intracellular messenger coupling LPS activation to EP synthesis, a role it plays in many other systems of hormone secretion. However, Ca<sup>2+</sup> is clearly required for other, as yet undefined processes in the production of EP. In some systems where calcium is thought to be important in stimulus-secretion coupling, Ca<sup>2+</sup> ionophores have been used to elicit a response by raising intracellular Ca<sup>2+</sup> levels. Thus, the ionophore A23187 has been shown to stimulate the release of histamine from mast cells [28] and the release of catecholamines from adrenal medulla [29]. We found that A23187, in doses that allowed influx of a radioactive Ca<sup>2+</sup> tracer, was not a sufficient stimulus for EP production by rabbit monocytes. Similarly, Foreman et al. reported that A23187 did not stimulate lysosomal enzyme secretion by macrophages [30], and McMillan et al. found that A23187-induced release of the lysosomal acid hydrolase B-N-acetylglucosaminidase from macrophages was accompanied by release of lactate dehydrogenase, a cytosolic enzyme, signifying the loss of cell integrity [31]. Our observation that EP production induced by LPS was reduced in the presence of A23187 may have been due to a similar toxic effect of the ionophore on monocytes. Alternatively, the presence of ionophore and a low Ca<sup>2+</sup> extracellular medium may have caused depletion

TABLE 5  
 Mean Fevers Produced by Supernatants from  $2 \times 10^6$  Monocytes  
 Incubated with 1mM B-cGMP, 1 mM IBMX, or 100 ng/ml PMA  
 With or Without 500 u/ml PMB

Potential Activators	$\Delta T (\pm SE)$ ( $^{\circ}C$ )
PMA (24-hour incubation)	$0.13 \pm 0.03$
(48-hour incubation)	$0.13 \pm 0.01$
B-cGMP	$1.17 \pm 0.12$
B-cGMP + PMB	$0.18 \pm 0.06$
IBMX	$0.40 \pm 0.08$
IBMX + PMB	$0.07 \pm 0.02$
IBMX + PMB + B-cGMP	$0.08 \pm 0.04$

of intracellular  $Ca^{2+}$  stores as cells were stimulated by LPS and released their intracellular pools. Hand et al. reported that incubation of alveolar macrophages with A23187 resulted in a rise in intracellular acid phosphatase and an increased rate of intracellular bactericidal activity, but only after 40 to 72 hours of incubation, and only in the presence of mM  $Mg^{2+}$  [32]. We found that  $Mg^{2+}$ , like  $Ca^{2+}$ , was ineffective in stimulating EP production with A23187. Since detectable EP is released in the first two to three hours after stimulation of the monocyte [33], it is unlikely that prolonging incubations would yield a different result.

While we were unable to demonstrate either influx or efflux of  $^{45}Ca^{2+}$  from LPS-stimulated monocytes, this does not exclude the possibility that transient rises of free cytoplasmic  $Ca^{2+}$  occur during stimulation. Changes in plasma membrane permeability allowing influx of  $Ca^{2+}$  that were matched by increased activity of the membrane-bound  $Ca^{2+}$  pump, or release of intracellular stores with rapid re-uptake would not necessarily cause changes in cell-associated  $Ca^{2+}$  observable in our experiments. This would require a system in which intracellular  $Ca^{2+}$  was very tightly controlled. The lack of increase in cell-associated  $Ca^{2+}$  during stimulation is consistent with the observation of Wood that suppression of EP production was associated with increased total  $Ca^{2+}$  levels in secreting cells [16].

Adenosine 3',5' cyclic monophosphate (cAMP) has been reported to inhibit macrophage migration [34], to decrease secretion of plasminogen activator by mouse exudate cells [35] and a macrophage-like tumor cell (J774.2) [36], and to decrease the secretion of lysosomal enzymes by mouse exudate cells [31]. We found that db-cAMP, which is more resistant to phosphodiesterase degradation and crosses membranes more readily than cAMP did not stimulate EP production in the presence of polymyxin B (PMB), an antibiotic which blocks the pyrogenicity of LPS *in vitro* [19]. Furthermore, addition of db-cAMP to incubations did not alter the production of EP in response to a standard dose of LPS. Epinephrine and prostaglandin  $E_1$ , in doses greater than those needed to stimulate adenylate cyclase maximally in macrophage homogenates [37], also failed to initiate or affect EP production. Staphylococcal enterotoxin B, another agent thought to activate adenylate cyclase, did not generate EP in the presence of PMB, confirming earlier observations that it did not stimulate EP production *in vitro* [38].

Other studies have shown that cGMP either decreases [39] or increases [40] human monocyte chemotaxis without affecting macrophage response to macrophage inhibi-

tory factor (MIF) [34]. We found that B-cGMP, with or without the phosphodiesterase inhibitor IBMX, did not stimulate EP production in the presence of PMB. There is an earlier brief report that IBMX does not affect LPS-stimulated EP production [41]. PMA, an agent thought to stimulate endogenous cGMP production, did not stimulate EP production when present in doses sufficient to evoke the metabolic oxygen burst.

Unlike earlier work with peritoneal exudate cells, in which preformed pyrogen was released in response to intracellular changes in Na<sup>+</sup> and K<sup>+</sup>, unstimulated blood cells require *de novo* synthesis of RNA and protein for EP production [42,43] with uptake of amino acids and incorporation into the new product [44]. In the studies reported here we have therefore examined the conditions necessary to initiate the production of EP, as well as its release. Recent work has shown that phagocytosis is not a necessary or sufficient stimulus for EP production, and that an intact cytoskeletal system is not required [45]. Steroids block EP production, but specific inhibitors of prostaglandin synthesis do not [46]. On the other hand, generation of leukotrienes may be important in the initial stages of cell stimulation with a known activator, though their possible role as exogenous stimuli for EP production has not been studied [47].

Given the capacity of all vertebrates, including poikilotherms, to produce endogenous pyrogen and the major role Interleukin-1 plays in stimulating the immune response directly by interacting with effector cells and indirectly by causing hyperthermia, it is interesting to find that pyrogen production is not controlled by the second messengers operating in most biological systems. Future work to define the mechanism of pyrogen production may reveal the existence of another messenger vital to this important controller of immune function.

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